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Review Article

Intestinal Peptide Transport Systems and Oral Drug Availability

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The intestinal peptide transport system has broad substrate specificities. In addition to its physiological function of absorbing di- and tripeptides resulting from the digestion of dietary proteins, this transport system also absorbs some orally administered peptidomimetic drugs, including β-lactam antibiotics, angiotensin converting enzyme inhibitors, renin inhibitors, bestatin, thrombin inhibitors, and thyrotropin-releasing hormone and its analogues. There have been several studies on the mechanism and substrate structure-affinity relationship for this transport system. Rapid progress has been made recently in studies on the molecular basis of the intestinal peptide transport system. A protein apparently involved in peptide transport has been isolated from rabbit small intestines, and genes for human intestinal peptide transporters have been cloned, sequenced and functionally expressed. This review summarizes these studies and addresses the pharmaceutical potential of the intestinal peptide transport system.

KEY WORDS: peptide transport; drug absorption; intestine; PepT1; HPT-1; PepT2; PHT-1.

INTRODUCTION

Successful drug development requires not only optimization of specific and potent pharmacological activity, but also efficient drug delivery to the target site. Many drug candidates fail to reach their therapeutic potentials due to poor bioavailability. For orally administered drugs, the challenge of reaching their sites of action is even greater because they must first cross the intestinal epithelial cells to get into the circulation system. Most oral drugs currently on the market rely on passive diffusion to cross cell membranes. More recently, however, it was realized that some orally administered peptidomimetic drugs are absorbed through the intestinal peptide transport system (1–7). Meanwhile, the rapid development in biotechnology and peptide synthesis, as well as new screening strategies have led to the exploitation of the unique pharmacological activities of peptide and peptidomimetic drugs. These developments have spurred great interest in the intestinal peptide transport system, as evidenced by the recent exponential increase in the number of publications on this topic. An appealing idea for oral drug delivery is to adapt the structures of drug molecules to the substrate structure features required by the intestinal peptide transport system for optimal molecular recognition. For the rational design of orally active drugs, an understanding of the mechanism of absorption from the gastrointestinal tract is essential.

MECHANISM OF INTESTINAL PEPTIDE TRANSPORT

The currently accepted model for intestinal transepithelial peptide transport is shown in Fig. 1. According to this model, the proton gradient and the membrane potential provide the driving force for peptide uptake into intestinal epithelial cells via proton-dependent peptide transporters located in the apical membranes. Peptides that are resistant to hydrolysis by intracellular peptidases are transported across the basolateral membrane via less well studied basolateral peptide transporter(s) (8–10). The Na^+/H^+-exchanger generates and maintains the inward proton gradient on the lumenal surface, while the Na^+/K^+-ATPase present in the basolateral membrane maintains a low intracellular sodium concentration. Because protons are co-transported with peptides across the epithelial membrane, this system is also referred to as H^+-dependent peptide cotransport system.

The substrate specificity of the intestinal peptide transport system has been determined based primarily on competition experiments. Most of these studies use radiolabeled dipeptide substrates to measure the transporter activity. The uptake of other compounds is assessed by their ability to inhibit the uptake of the dipeptide substrate. However, some compounds can bind to the peptide transporters and therefore inhibit substrate transport without necessarily being transported themselves (11,12). For example, although cephradine undergoes carrier-mediated

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ABBREVIATIONS: ABC, ATP-binding cassette; ACE, angiotensin converting enzyme; BBMV, brush border membrane vesicles; cDNA, complementary DNA; cRNA, complementary RNA; CHO cells, Chinese hamster ovary cells; GI, gastrointestine; mAb, monoclonal antibody; TAP, transporter associated with antigen processing; TI, thrombin inhibitor; TM, transmembrane domains.

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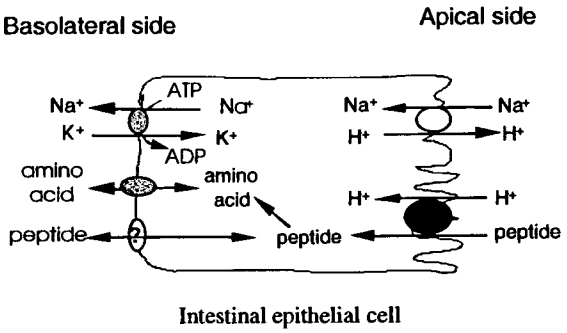


Fig. 1. Currently accepted model for intestinal transepithelial peptide transport Adapted from (118).

transport by peptide transporters and benzylpenicillin does not (13), both compounds inhibit cephalexin uptake with similar capacity (14). This is partly why no definitive structure-transport relationships have been postulated, despite continuing discovery of new compounds showing affinity for the peptide transporters. Also contributing to the lack of clearly defined structure-activity relationships may be the fact that some substrates for the intestinal peptide transport system are also substrates for other transport systems such as the monocarboxylic acid transport system (15). Nevertheless, these studies have provided useful information on the structure requirements of substrates for the intestinal peptide transport system. The consensus from studies on the substrate specificity of the intestinal transport system is that the following structural features appear to be required by the intestinal peptide transport system: a free C-terminal carboxyl group or a group capable of hydrogen bond formation, either an amino group or a weakly basic group at the N-terminus, an overall charge of less than two positive units, and preferably a C-terminal amino acid in its L-configuration. Figure 2 shows the essential substrate structure for the intestinal peptide transport system as proposed by Kramer and colleagues (16). However, some compounds, including the renin inhibitors, do not completely fit into this structure. A fuller understanding of the molecular features recognized by the peptide transporters will be important for rational design of drug molecules which can be absorbed via this route. The cloning, expression, and characterization of individual peptide transporters (described below) will give additional insight into the structural requirements of specific transporters.

PHARMACEUTICAL IMPORTANCE

The discovery of the intestinal peptide transport system and its broad substrate specificity has led to a new approach

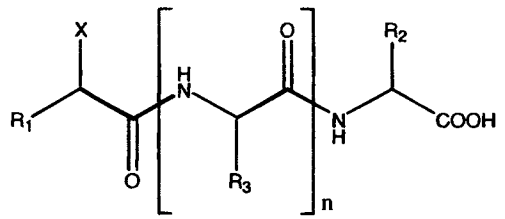


Fig. 2. Hypothetical substrates for the intestinal peptide transport system. n: 0 or 1. R_1 and R_3: unclear. R_2: H, CH_3, vinyl, or other small, electrically neutral group that does not have steric hindrance of the free carboxyl group. X: a group capable of accepting protons, such as NH_2, imidazolyl, or thiol group. Adapted from (16).

for intestinal drug absorption. The strategy is to synthesize peptide analogues that are small in size, metabolically stable and recognizable by the intestinal peptide transport system.

Many orally active peptide drugs share structural features with physiologic substrates of the peptide transport system. The intestinal peptide transport system accepts these drug molecules as substrates and acts as a carrier for their effective absorption (17). The β-lactam antibiotics were the first peptide drugs identified as substrates for the intestinal peptide transport system (18–22). These antibiotics are hydrophilic weak acids containing a peptide bond and are generally ionized at the pH of the intestinal contents. On the basis of their chemical and physicochemical properties, cephalosporins would be expected to be poorly absorbed from the intestines. However, pharmacokinetic studies showed that many of them are well absorbed (23). Following the identification of cephalosporins and penicillins as substrates for the peptide transport system, the absorption of several angiotensin converting enzyme (ACE) inhibitors (5,6,24,25), and renin inhibitors (4) were found to be mediated through the intestinal peptide transport system.

Drug transport via the peptide transport system is a saturable and concentration dependent phenomenon which is characterized by the Michaelis-Menten constants K_m and J_{max} (or V_{max}). Detailed transport parameters are available for several hydrolysis resistant peptides β-lactam antibiotics and ACE inhibitors (Table 1). The transport parameters are the permeability parameters P_c and P_m (for carrier-mediated and passive diffusion transport pathways, respectively), the maximal transport rate V_{max} (or J_{max}) and the Michaelis-Menten constant, K_m. The parameters in this table were determined by nonlinear regression of the data obtained from *in situ* single-pass rat intestinal perfusion experiments to either of the following alternative equations (26):

$$P_w = J_{max}/(K_m + C_w) + P_m$$

or

$$P_w = P_c/[1 + (C_w/K_m)] + P_m$$

where $P_c = J_{max}/K_m$, P_w is the intestinal wall permeability, and C_w is drug intestinal wall concentrations usually expressed in mM.

For the β-lactams in Table 1, the carrier-mediated permeability parameter, P_c, is relatively constant while J_{max} and K_m vary significantly. The plasma concentrations of several β-lactams have been measured after oral administration (27). The maximal flux or the carrier capacity, J_{max}, was found to have the highest impact on the absorption process and subsequent plasma levels. High capacity, low-affinity β-lactams are better absorbed. For example, cephradine, cephalexin and cefadroxil all have good oral availability, while cefixime is less well absorbed (Table 1).

The "peptide" nature of the substrates for the peptide transport system is revealed by a comparison of their chemical structures to di- or tripeptides (Fig. 3). For example, cephalexin resembles the structure of the tripeptide Phe-Cys-Val, and enalapril resembles the tripeptide Phe-Ala-Pro. The β-lactams and ACE inhibitors are di- or tripeptide analogues having at least one peptide bond and a free terminal carboxyl group. Although a second peptide bond and an amino terminal or an α-amino group exist in most β-lactams as in tripeptides, it is not a

Table 1. Summary of Permeability Data for Some Dipeptides and Peptide Drugs β-Lactams and ACE Inhibitors

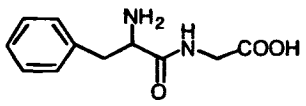
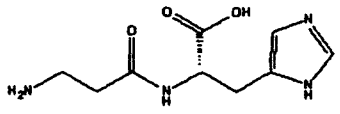
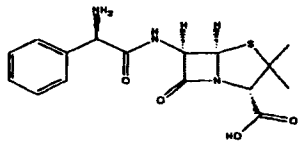
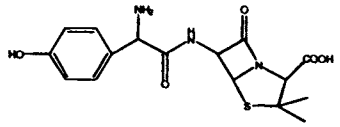
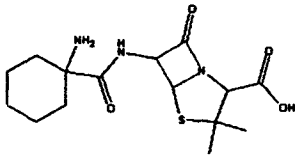
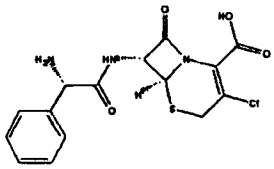
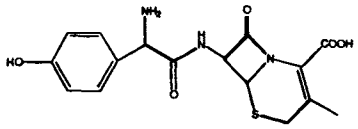
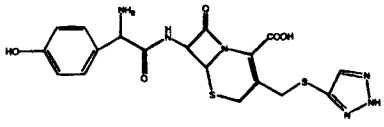
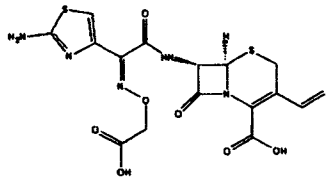
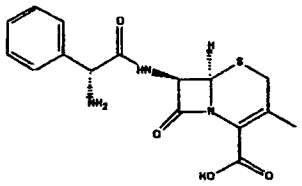
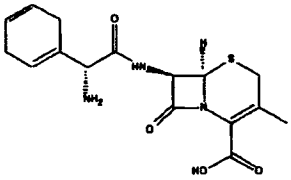
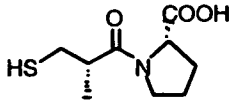
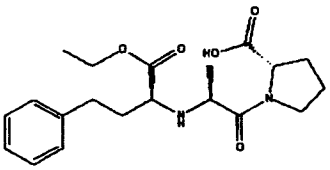
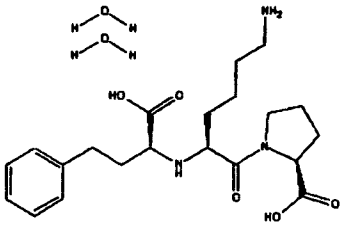
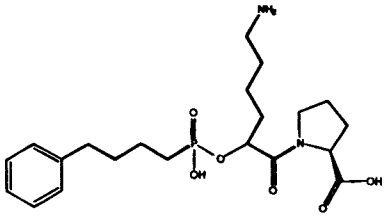
Compound		K_m (mM)	J_{max} (nmol/cm^2/min)	P_c (μl/cm^2/min)	P_m (μl/cm^2/min)
Peptides					
Phe-Gly (222.2)		1.29	6.87	5.33	—
Carnosine (226.2)		12.9	6.62	0.51	—
β-Lactams					
Ampicillin (349.4)		15.8	11.8	0.75	0.0
Amoxicillin (365.4)		0.058	0.044	0.558	0.76
Cyclacillin (341.4)		14.0	16.3	1.14	0.0
Cefaclor (367.8)		16.1	21.3	1.32	0.0
Cefadroxil (363.4)		5.9	8.4	1.43	0.0
Cefatrizine (462.5)		0.58	0.73	1.25	0.20
Cefixime (453.4)		0.031	0.016	0.184	0.52

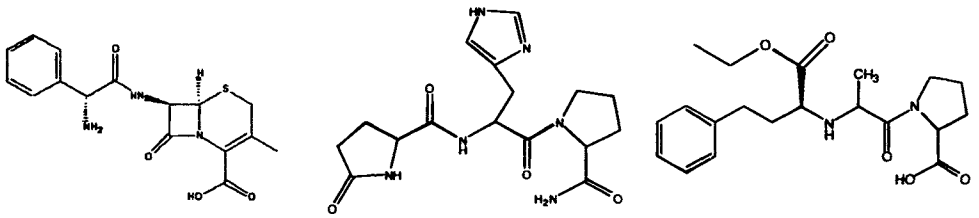
Table 1. Continued

Compound		K_m (mM)	J_{max} (nmol/cm²/min)	P_c (μl/cm²/min)	P_m (μl/cm²/min)
Cephalexin (347.4)		7.2	9.1	1.30	0.0
Cephradine (349.4)		1.48	1.57	1.06	0.30
ACE inhibitors					
Captopril (217.3)		5.9	12.3	2.08	1.0
Enalapril (376.4)		0.07	0.13	1.9	0.35
Lisinopril (405.5)		0.082	0.032	0.39	0.0
SQ 29,852 (440.5)		0.08	0.16	2.0	0.25

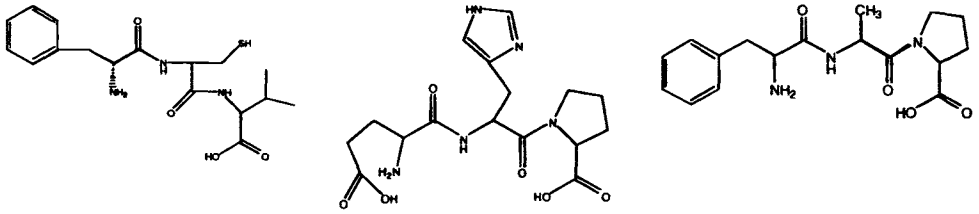
Note: K_m, Michaelis-Menten constant; J_{max}, maximal transport rate (flux at steady state $J_m = P_w \cdot C_w$); $P_c = J_{max}/K_m$, permeability via carrier-mediated transport; P_m, permeability through passive diffusion; molecular weight in parenthesis.
* Data taken from (40).

prerequisite for the peptide transport system. For example, cefixime does not have an α-amino group and SQ 29,852 does not have a second peptide bond (1,6). Yet these compounds are absorbed via the peptide transport system.

Other groups of bioactive compounds absorbed via this transport system are bestatin (28,29), thrombin inhibitors (30) and thyrotropin-releasing hormone (TRH) and its analogues (Fig. 4) (31). Bestatin is a dipeptide containing an unusual amino acid (Fig. 4). It has antitumor activity and has been used clinically as an anticancer agent in p.o. dosage form. The uptake of bestatin into brush border membrane vesicles (BBMV) was stimulated by an inwardly directed H^+-gradient and by an interior-negative membrane potential (28). The uptake was inhibited by dipeptides and cephalosporins, but not by amino acids.



Peptidyl Mimetics	Cephalexin (MW: 347.4)	TRH (pyro-Glu-L-His-L-Pro-NH_2) (MW: 362.4)	Enalapril (MW: 376.4)



Peptides	D-Phe-Cys-Val (MW: 367.5)	Glu-His-Pro (MW: 381.4)	Phe-Ala-Pro (MW: 333.4)

Fig. 3. Comparison of structures of peptidyl mimetic drugs and peptides.

The apparent K_m and J_{max} were estimated to be 0.52 mM and 7.7 nmol/min/mg protein in BBMV, respectively (28).

A number of protein inhibitors of thrombin are available for the treatment of thrombotic disorders. However, drug administration is a serious problem. Therefore, low molecular-weight peptidomimetic thrombin inhibitors have been developed as anticoagulants with peroral activity. Thrombin inhibitor (TI) shown in Fig. 4 is a derivative of L-Asp-D-Phe. It fulfills the minimal structural requirements with a free carboxyl group and one amide group. Its uptake through Caco-2 cell monolayers can be inhibited by dipeptides and β-lactams. The transport parameters are: K_m of 1.67 mM and J_{max} of 0.026 nmol/min/mg protein per min in BBMV (30). When the carboxyl group was esterified with bulky side chains, both transporter-mediated uptake in Caco-2 monolayers and oral availability were dramatically reduced (up to 10-fold). It is not clear, however, whether paracellular transport also plays a significant role in the permeability of this thrombin inhibitor across Caco-2 monolayers.

Thyrotropin-releasing hormone (TRH) is a tripeptide (pyro-L-Glu-L-His-L-Pro-NH_2) (Fig. 4) produced by the hypothalamus. It has a variety of endocrine activities as well as central nervous system-related biological activities. TRH has attracted attention as a potential drug for the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma, schizophrenia and Alzheimer's disease (32–35). TRH shows a transporter-mediated uptake component, which operates parallel to a passive pathway in Caco-2 cells (36). At low TRH concentrations (<3 mM), active transport becomes prominent. The uptake is optimum at pH 6 and decreases considerably at pH 7.4. The transport parameters of the active transport component are: K_m of 1.59 mM and J_{max} of 0.002 nmol/mg of protein per min in BBMV (36). Although the uptake of TRH has a transporter-mediated component, and orally administered TRH has been reported to enhance thyroid stimulating hormone release in human (37,38) and the transport efficiency of TRH across the intestines is relatively low compared to other di-/tripeptides and some β-lactam antibiotics (39). It is believed that the lack of C-terminal carboxyl groups and the presence of an intramolecular γ-linkage reduce its affinity to the peptide transporters.

The intestinal peptide transport system has been a key target for prodrug approaches. According to this approach, prodrugs appropriately designed in the form of di-/tripeptide analogues can be absorbed across the intestinal brush border

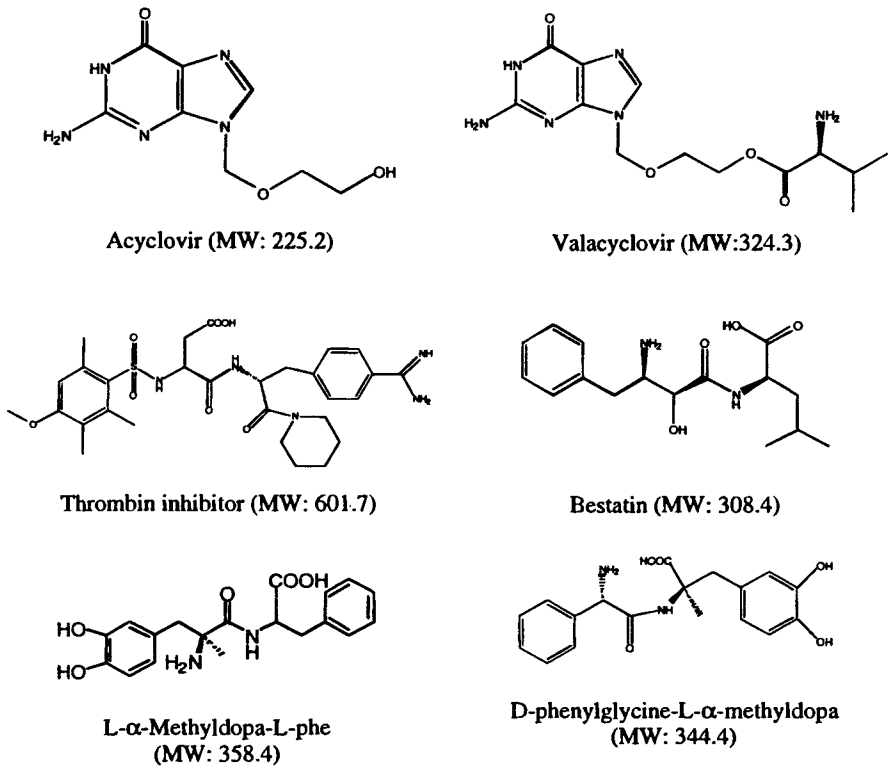


Fig. 4. Peptidyl drugs that are absorbed via the intestinal peptide transport system.

membrane via the peptide transport system. The prodrug may be targeted to the epithelial membrane and hydrolyzed on the apical surface of the epithelium prior to absorption by the transporter or may be absorbed intact and hydrolyzed intracellularly by peptidases or esterases prior to exit from the cell (40).

L-α-Methyldopa is a poorly absorbed antihypertensive agent and an amino acid analogue. Its absorption is mediated through an amino acid transporter. Amino acid transporters are structurally restrictive (41) and this is thought to be the main reason for the poor intestinal absorption of L-α-methyldopa since the α-methyl group severely hinders the binding of the substrate to the transporter. Attempts have been made to improve the absorption of L-α-methyldopa by making its dipeptide prodrugs in order to use the more accommodating peptide transport system.

Hu and colleagues (42) prepared a dipeptidyl derivative of L-α-methyldopa: L-α-methyldopa-L-phenylalanine (Fig. 4). This prodrug displayed up to 20 times increase in intestinal permeability compared to the parent compound, L-α-methyldopa, in *in situ* single pass rat intestinal perfusion studies. The uptake of L-α-methyldopa-Phe was inhibited by dipeptides and cephradine (42,43). Hydrolysis of the dipeptidyl prodrugs was observed in intestinal cell homogenates *in vitro*, suggesting liberation of the parent compound after intestinal uptake. Further studies indicated an increase in oral bioavailability of L-α-methyldopa after administration of the prodrugs in the small intestine of rats (44).

Wang and colleagues (45) prepared another dipeptidyl derivative of L-α-methyldopa: D-Phenylglycine-L-α-methyldopa (Fig. 4). In *in situ* single pass rat jejunal perfusion studies, this compound demonstrated a 3.5-fold increase in permeability compared with that of L-α-methyldopa, indicating that this prodrug was better absorbed in the intestine than its parent compound. In the presence of an inward proton gradient, the prodrug showed Michaelis-Menten type saturable kinetics of BBMV uptake with a K_m of 0.06 mM and V_{max} of 4.4 nmol/min/mg protein. The high affinity and the high value of V_{max}/K_m suggest that the peptide transporter is the major mechanism for uptake. The uptake of the prodrug was significantly inhibited by cephradine and dipeptides Gly-L-Pro and Gly-L-Phe. These results demonstrated the feasibility of using the peptide transport system to improve the intestinal absorption of pharmacologically active amino acid analogues.

This strategy has been extended to the absorption of the nucleoside antiviral compound acyclovir (ACV) which has poor oral bioavailability. When the 5′-amino acid ester prodrug of ACV was prepared there was increased excretion in rats (46). Studies using Caco-2 monolayers indicated that absorption from the mucosal to the serosal side were enhanced 7-fold by conjugation with L-valine and that uptake was inhibited by the dipeptide glycylsarcosine and not L-valine. There also was stereospecific preference for the L-valine ester over the D-valine ester. Transport across the monolayers saturated with increasing concentrations of the conjugate with a K_m of about 300 μM for the mucosal-to-serosal flux (46). Furthermore, the conjugate inhibited the uptake of cephalexin, a known substrate of the peptide carrier. Thus, uptake appeared to be mediated by the peptide transporter. These studies have been extended further using Chinese hamster ovary cells or HeLa transfected with the human peptide transporter, PepT1 described below (46,47). Uptake of Gly-Sar was inhibited by L-Val-ACV with less well by D-Val-ACV with a 3-fold difference in their IC_{50}s. Similar results were obtained with the L-valyl ester prodrug of

zidovudine (AZT). Since these compounds do not contain a peptide bond, these studies indicate that the utility of the peptide transporter for drug absorption extends well beyond peptide delivery.

THE MOLECULAR BASIS OF INTESTINAL PEPTIDE TRANSPORT SYSTEM

Rapid progress has been made in studies on the molecular basis of the intestinal peptide transport. A protein apparently involved in peptide transport has been isolated from rabbit small intestines and genes for human intestinal peptide transporters have been cloned, sequenced and expressed.

Isolation and Characterization of a Peptide Transporter from Rabbit Small Intestine

Photoaffinity labeling studies using benzylpenicillin as photolabile ligand suggest that an integral membrane protein with an apparent molecular weight of 127 kDa is directly involved in the dipeptide transport activity in rabbit small intestine (48). This protein can be completely solubilized from intestinal brush border membrane vesicles with nonionic detergents such as Triton X-100, n-octyl-glycoside or CHAPS. Purification to over 95% homogeneity was achieved by lectin-affinity chromatography with wheat germ lectin followed by ion-exchange chromatography (48,49). Antibodies prepared against the purified protein were able to inhibit the transport of cephalexin and photoaffinity labeling of the 127 kDa protein in intact BBMV. A direct role of this protein as the intestinal peptide transporter was demonstrated by reconstitution into liposomes (49). In reconstituted liposomes, transport activity of the isolated protein was restored and showed the same substrate specificity as in BBMV. Furthermore, the uptake of cephalexin into reconstituted liposomes was stereospecific and was stimulated by an inwardly directed H^+-gradient, whereas pure liposomes without incorporated protein did not show a H^+-dependent cephalexin uptake.

The purified transport protein has been partially sequenced after chemical and enzymatic fragmentation (16). No sequence homology was found to any known proteins. However, the purified protein was immunoprecipitated with polyclonal antibodies raised against the 127 kDa protein, sucrase activity was coprecipitated. When the functional molecular mass of the 127 kDa β-lactam binding protein was assessed by target size analysis a mass of 165 kDa was obtained. This suggests the protein exists as a homo- or heterodimeric structure (50). With immunohistochemical methods, the 127 kDa protein was identified in the jejunum and the proximal tubule of the kidney, whereas no immunostaining was found in the colon. In the jejunum and the kidney, this peptide transporter was exclusively expressed in the brush-border membrane of the epithelial cells and was completely absent in the basolateral and lateral membranes (16).

Molecular Cloning and Expression of PepT1

Isolation of proteins which are responsible for the transport of various organic solutes across animal cell membranes using conventional protein purification procedures has in general been found to be difficult. Several research groups have therefore used alternate strategies to study peptide transporter, in particular the so-called 'expression cloning.' This strategy involves the use of *Xenopus laevis* oocytes to express a particular transport system in a functionally competent form upon microinjection of exogenous mRNA into the oocytes. *Xenopus laevis* oocytes are known to be capable of not only translating injected mRNA from eukaryotic sources, but also carrying out posttranslational modifications and targeting.

Microinjection of poly(A)$^+$ mRNA mixtures prepared from rabbit intestinal mucosal cells led to the successful expression of intestinal peptide transporters in *Xenopus laevis* oocytes (51). This work was followed by the functional expression of rat (52) and human intestinal peptide transporters (53) in *Xenopus laevis* oocytes. Size fractionation of mRNA prior to injection into the oocytes indicated that the functional expression of the peptide transport activity is associated with mRNA 1.8–3.6 kb in size (22,52). These studies demonstrated the feasibility of cloning the intestinal peptide transporters using this expression cloning strategy.

Using the expression cloning strategy and screening a rabbit intestinal cDNA library for the uptake of ^{14}C-labeled dipeptide glycylsarcosine (Gly-Sar), Fei and colleagues (54) isolated a rabbit intestinal cDNA which codes for a 707 amino acid peptide transporter. The protein encoded by this gene was designated PepT1. Using the same technique, Boll and colleagues reported independently the cloning of the same gene (55). The isolation of rabbit PepT1 cDNA has led to the isolation of PepT1 cDNA from human (56) and rat (57,58) intestinal cDNA libraries using probes derived from rabbit PepT1 cDNA.

Not surprisingly, PepT1 cDNAs from human, rabbit, and rat encode highly homologous proteins containing 708 (human), 707 (rabbit) or 710 (rat) amino acids, respectively (54,56–58). The protein core was predicted to have a relative molecular mass of approximately 79 kDa and an isoelectric point of 8.6. The protein contains 12 putative transmembrane spanning domains (54). PepT1 from all three species contains multiple N-glycosylation sites and are heavily glycosylated. There is a single potential site for protein kinase C-dependent phosphorylation in rabbit and rat PepT1, while human PepT1 contains two potential sites for protein kinase C phosphorylation. Rabbit and rat PepT1 also contain a potential site for protein kinase A-dependent phosphorylation. Interestingly, human PepT1 does not possess any site for protein kinase A. Chromosomal assignment studies with somatic cell hybrid analysis and *in situ* hybridization have shown that the human PepT1 gene is located on the human chromosome 13 q33Æq34 (56).

PepT1 cDNA has been functionally expressed in *X. laevis* oocytes by microinjection of the complementary RNA (cRNA) (54) or in HeLa cells using a viral transfection system (56). A cell line stably transfected with the human PepT1 cDNA has also been established from Chinese hamster ovary cells (59). Cells expressing PepT1 acquired the ability to transport a variety of di- and tripeptides as well as some peptidomimetic drugs, but not free amino acids. The transport was eletrogenic and coupled to an inward proton gradient (60). Functional expression studies have also established that PepT1 is a low-affinity transporter, with K_m in the millimolar range. Site specific mutagenesis of tyrosine 167 located within transmembrane spanning domain TMD 5 and tryptophan 294 (TMD 7) and glutamate 595 (TMD 10) renders a transporter with greatly reduced ability to take up Gly-Sar when expressed in HEK 293 cells, suggesting these are critical amino acids important in function in the native transporter (61,62). Furthermore, PepT1 is proposed to form a

channel through which substrates are transported (62). Studies of the substrate specificity have been performed by expressing rabbit PepT1 in the yeast *Pichia* and screening a wide variety of compounds to determine a common pharmacophore (63). The results indicate that the need for a zwitterionic amino acid functionality separated by a molecular distance of 500 to 630 pm between two charged centers. Studies conducted with ω-amino fatty acids, ω-AFA, indicated that this is a substrate of PepT1 and both the amino and the carboxylic acid group are required for electrogenic transport although removal of one charged group still permits binding (63). Thus, PepT1 apparently transports a wider array of substrates than previously believed.

In situ hybridization with PepT1-specific probes has shown that the transporter is expressed all along the small intestine (64). PepT1 mRNA is also expressed in liver (54), kidney (65), pancreas (66) and at a very much reduced level in colon, but is absent in the stomach or caecum (64). In the small intestine, the expression of PepT1 is limited to the mucosal cells. Along the crypt-villus axis, the PepT1 mRNA is not detectable in the crypt. It starts to appear at the crypt-villus junction, and increases rapidly towards the villus tip.

In one hybrid depletion experiment, rabbit small-intestine poly(A)$^+$ RNA was incubated with antisense oligonucleotide corresponding to the 5′-end coding region of PepT1 cDNA before being injected into *X. laevis* oocytes (54). The antisense oligonucleotide completely suppressed the uptake of Gly-Sar by the oocytes. This result demonstrates that PepT1 cDNA is responsible for the expressed peptide transport of Gly-Sar, suggesting that PepT1 may be the dominate peptide transporter for Gly-Sar in enterocytes. However, since all genes do not transcribe at the same time in a cell and all mRNA do not translate under the same conditions (67,68), the antisense suppression experiment cannot rule out the existence of other peptide transporters in enterocytes.

In vitro translation of PepT1 cRNA using rabbit reticulocyte lysates in the presence of microsomes yielded a translation product of apparent molecular mass of 71 kDa. Treatment with endoglycosidase H revealed that at least 11 kDa of the 71 kDa was due to N-linked glycosylation, which suggests N-glycosylation at multiple sites. Intriguingly, the 60 kDa for the protein moiety is lower than the calculated molecular mass of 79 kDa for PepT1 based on its predicted amino acid sequence.

Purification of peptide transporter(s) from the rat intestine was accomplished by extraction with n-octylglucoside and ceftibuten-affinity chromatography (69). The proteins were reconstituted in liposomes and shown to possess proton-dependent uptake of ceftibuten. Two proteins were identified of molecular weights of 117 kDa and 127 kDa. When N-glycanase digested the smaller protein yielded a protein of ~79 kDa consist with the calculated molecular weight for rat PepT1.

Another protein may be associated with PepT1. Saito and colleagues screened a human duodenum cDNA library with a probe derived from rabbit PepT1 cDNA (70). They identified a cDNA encoding a pH-sensing regulatory factor protein, designated as hPepT1-RF (70). The deduced primary sequence of this protein has 208-amino acids and is virtually identical to the N-terminal region of PepT1. It has no transport activity by itself, but appears to reduce the uptake of Gly-Sar by PepT1 and shift the optimal pH of PepT1 from ~5.5 to ~6.0. This small protein has five predicted transmembrane spanning domains based on hydropathy analysis. It does not have a potential glycosylation site nor any potential regulatory site in its amino acid sequence. This is the first description of a regulatory factor that modulates the activity of the peptide transporter and its physiological importance remains to be further elucidated.

Molecular Cloning and Expression of HPT-1

Published almost simultaneously with rabbit PepT1, Dantzig and colleagues reported the identification of another peptide transporter, which was termed as HPT-1 (the Human intestinal Peptide Transporter-1) (71). The strategy employed in the discovery of HPT-1 and the cloning and expression of its cDNA is different from the expression cloning strategy described in the discovery of PepT1. While PepT1 was cloned by expression cloning from a rabbit small intestinal cDNA library, HPT-1 was cloned using a functionally inhibitory monoclonal antibody.

Polyclonal mouse antibodies were induced using Caco-2 cell membrane protein mixture as an immunogen. The mouse spleen cells produced antibodies against all Caco-2 cell membrane proteins including peptide transporters. Monoclonal antibodies were then identified using an uptake blocking assay. Antibodies that blocked ^{14}C-cephalexin uptake by Caco-2 cells were the target antibodies. Monoclonal antibody (mAb) 13G6 had the strongest blocking ability, and was used in Western blot assays of Caco-2 cell membrane proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE). A single protein band with an apparent molecular weight of 120 ± 10 kDa was detected. Deglycosylation with endoglycosidase F indicated that about 20 kDa of this protein was from N-linked oligosaccharide chains. Western blot assays of other human cell lines identified this protein in cells derived from the gastrointestinal (GI) tract only, including Caco-2, HT-29, and COLO 320 cells. Immunohistochemical staining with mAb 13G6 showed that this protein was present in every segment of the intestinal tract as well as the pancreatic ducts, but was absent in all other tissues tested. In Caco-2 cells, this protein appears to be located on the apical surface (71).

Caco-2 cDNA libraries were screened with mAb 13G6 and a clone of 3345 base pairs (bp) was identified. It contains an open reading frame (ORF) of 2496 bp, which was designated *hpt-1*. The putative protein encoded by *hpt-1* contains 832 amino acids. In contrast to PepT1, the calculated mass of HPT-1 is consistent with the apparent size of the deglycosylated protein. HPT-1 has seven potential N-glycosylation sites, which is in agreement with the reduced molecular weight of HPT-1 after glycosidase F treatment. Analysis of the deduced amino acid sequence of HPT-1 indicated that this protein shares several conserved structural elements with the cadherin superfamily of calcium-dependent cell-cell adhesion proteins, although the overall homology is only 20–30%. One transmembrane domain was predicted based on Kyte-Doolittle hydropathy analysis (71). However, up to 6 transmembrane domains were predicted based on statistical analysis of a database of naturally occurring transmembrane proteins (72)

HPT-1 and PepT1 do not share significant homology and are not related to each other. Human PepT1 and HPT-1 share only 16% identity and 41% similarity in their amino acid sequences (56). Furthermore, both proteins have no significant

sequence homology to the 127 kDa polypeptide Kramer and co-workers described (16) a result that suggest the existence of multiple peptide transporters in small intestinal brush-border membranes.

The *hpt-1* gene has been expressed in Chinese hamster ovary cells (CHO) (71). The expression of HPT-1 in these cells was confirmed by immunoblot analysis with mAb 13G6. The characteristics of the HPT-1 induced transport activity were similar to the endogenous peptide transport activity in Caco-2 cells. CHO cells transfected with the *hpt-1* gene showed two- to three-fold higher uptake of cephalexin than the control. The magnitude of increase in peptide uptake is in the same range as other cloned transporters. The uptake of cephalexin by the transfectant was dependent on an inwardly directed proton gradient and was inhibited by mAb 13G6. The uptake of the dipeptide bestatin was also examined. The transfectant showed over two-fold higher uptake of bestatin than the control. The uptake was inhibited by addition of cephalexin or another dipeptide, Gly-Pro, and was dependent on an inwardly directed proton gradient.

HPT-1 has been purified from Caco-2 cell membranes and human intestinal brush border membranes (73). The amino acid sequence of HPT-1 deduced from HPT-1 cDNA sequence has been confirmed with Edman degradation and tandem mass spectrometry. The purified HPT-1 was shown to actively transport cephalexin and *p*-hydroxyloracarbef after being reconstituted into liposomes.

Other Peptide Transporters

Peptide Transporters in Renal Epithelia

In addition to intestinal brush border membranes, epithelial cells in other tissues have also been found to contain proton-coupled peptide transporters. These tissues include kidney, lung, placenta, and brain.

Mammalian kidney expresses a transport system specific for small peptides. Using probes derived from rabbit PepT1 cDNA, H^+-coupled peptide transporters have been cloned from human (65), rabbit (74), and rat (75) kidney cDNA libraries. These renal peptide transporters are termed PepT2. They consist of 729 amino acids and share approximately 50% sequence identity with its intestinal counterpart PepT1. Functional studies have established that the renal peptide transport system is similar but not identical to PepT1. The substrate affinity of PepT2 is much higher than that of PepT1 and their substrate specificity differs (18). A chimeric protein containing the amino acid residues 1–401 of PepT2 and residues 402–707 of PepT1 was prepared (76); the chimeric protein retained the high affinity and substrate specificity of native PepT2, indicating that the amino-terminal portion of PepT2 is important in these functions (76). Since both PepT1 and PepT2 are expressed in the kidney, it has been speculated that these two transporters may be differentially expressed along the length of the nephron (77). The concentration of small peptides in the lumen of the nephron is likely to increase significantly along the length of the nephron because of the action of highly active brush border membrane peptidases such as dipeptidylpeptidase IV on larger peptides and proteins. The expression of the high-affinity transporter PepT2 in the proximal regions and the low-affinity transporter PepT1 in the distal regions would be advantageous under these physiologic conditions. Renal peptide transporters are believed to play a significant role in conserving amino nitrogen which might otherwise be lost in the urine.

The lung is derived from the foregut during embryonic development and it is therefore not surprising that transepithelial peptide transport activity has been found in the lung (78–80). Peptide uptake has been demonstrated with BBMV prepared from rat lung (80) and with monolayers of alveolar type II pneumocytes (81). The uptake process is electrogenic and dependent on an inwardly directed pH gradient. These results suggest that the lung may be a site for effective delivery of small, therapeutically active peptides.

Peptide Transporters in the Brain and Retina

A brain peptide transporter has been cloned and been shown to be identical to the renal peptide transporter PepT2 (82). When expressed in HeLa or SK-N-SH cells, brain PepT2 transports several peptides including N-acetyl-L-aspartyl-L-glutamate and the fluorescently tagged peptide β-Ala-lys-N_ϵ-AMCA. PepT2 is expressed by astrocytes (type 1 ad II) and O-2A progenitor cells but not neuronal cells or oligodendrocytes (83). Yamashita and colleagues reported the cloning and functional characterization of a peptide/histidine transporter (PHT1) in rat brain and retina using a probe derived from PepT1 cDNA (84). This transporter is predicted to contain 572 amino acid residues with 12 putative transmembrane-spanning domains. The amino acid sequence has moderate homology with a plant peptide/histidine transporter: NTR1 (also called AtPTR2-B) (85,86). When expressed in *Xenopus laevis* oocytes, PHT1 cRNA induced high affinity proton-dependent carnosine and histidine transport activity. This transport process was inhibited by dipeptides and tripeptides but not by free amino acids. With *in situ* hybridization analysis, PHT1 mRNA was found to distribute throughout the brain, especially in the hippocampus, choroid plexus, cerebellum, and pontine nucleus. PHT1 was believed to contribute to the uptake of neuromodulators and clearance of degraded neuropeptides (84).

Peptide Transporters in the Placenta

In the placenta, Vatish and colleagues (87) have confirmed the earlier findings of Ganapathy and colleagues (88) that oligopeptides are transported intact into isolated placenta BBMV. The uptake of peptides into human full-term placental BBMV were time- and temperature-dependent (89). The uptake of D-Phe-L-Ala was stimulated by an inwardly-directed proton gradient (89). The identity of the peptide transporter has not been determined.

Other Peptide Transporter Families

Besides the peptide transporters described above, most other currently known peptide transporters belong to the ABC-type (ATP-binding cassette) peptide transporter family (Table 2). In addition to the well known TAP1/TAP2 (transporter associated with antigen processing), this family include the multidrug resistance proteins (90,91), the yeast transporter for the farnesylated peptide pheromone a, STE6 (92), dipeptide transport systems (Dpp system) and oligopeptide transport systems (Opp system) from the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* (93–95), and peptide-transport

Table 2. Peptide Transporter Families

	Definition	References
PTR family of peptide transporters		
Human PepT1	Human intestinal peptide transporter	(56, 104)
Rabbit PepT1	Rabbit intestinal peptide transporter	(54, 55)
Rat PepT1	Rat intestinal peptide transporter	(57, 58)
Human PepT2	Human renal peptide transporter	(65)
Rabbit PepT2	Rabbit renal peptide transporter	(74)
Rat PepT2	Rat renal peptide transporter	(75)
Rat PHT1	Rat brain peptide/histidine transporter	(84)
AtPTR2-A	*Arabidopsis thaliana* peptide transporters (plants)	(105)
AtPTR2-B (NTR1)	*Arabidopsis thaliana* peptide transporters (plants)	(85, 86, 105)
AtCHL 1	*Arabidopsis thalina* nitrate transporter (plants)	(106)
ScPtr2	*Saccharomyces cerevisiae* peptide transporter (yeast)	(107)
CaPtr2p	*Candida albicans* peptide transporter (fungi)	(108)
DtpT	*Lactococcus lactis* di- and tripeptide transporter (bacteria)	(109-111)
ABC family of peptide transporters		
TAP1/TAP2	Transporter associated with antigen processing	(112)
AmiA, AmiC, AmiD, AmiE, AmiF	*Streptococcus pneumoniae* Ami oligopeptide transport system	(98)
DciaA, DciaB, DciaC, DciaD, DciaE	*Bacillus subtilis* DciA dipeptide transport system	(97)
EcDppA	*E. coli* dipeptide-binding protein	(113, 114)
EcOppA	*E. coli* oligopeptide-binding protein	(115)
LacOppA, LacOppB, LacOppC, LacOppD, LacOppF	*Lactococcus lactis* oligopeptide transport system	(116)
SalOppA	*Salmonella typhimurium* oligopeptide permease	(94, 95, 117)

systems from Gram-positive bacteria such as the *ami* locus of *Streptococcus pneumoniae* and *Lactococcus lactis* (96–100). In general, members of the ABC-transporter family in mammalian systems use intracellular ATP as the energy source to efflux the substrate.

PepT1 from human, rabbit, and rat share extensive amino acid sequence homology. Human PepT1 has over 80% amino acid sequence identity with rabbit and rat PepT1 (56,58). A significant sequence homology (approximately 50% identity and 70% similarity) exists between PepT1 and PepT2. PepT1 also shows some similarity (up to 25% identity) to a few other peptide transporters, including *Saccharomyces cerevisiae* peptide transporter (ScPtr2), *Arabidopsis thaliana* peptide transporters (AtPTR2), *Candida albicans* peptide transporter CaPtr2p, and *Lactococcus lactis* di- and tripeptide transporter DtpT. These peptide transporters are distinct from ABC-type peptide transporters. Therefore, a new family of peptide transporters has been suggested and has been called the PTR family (peptide transport) (Table 2) (101,102). Some PTR family members have been previously classified as members of the POT family (Proton-dependent oligopeptide transporters) (103).

HPT-1 has no significant homology with either ABC-type peptide transporters or the PTR family members (16% identity and 41% similarity with human PepT1 (56). Like HPT-1, the 127 kDa protein Kramer and colleagues described (16) does not cluster with any group of peptide transporters in amino acid sequence analysis. Furthermore, this protein appears to be unrelated with HPT-1 (16). Therefore, multiple peptide transporters exist in small intestinal brush-border membranes. This makes biological sense considering the number and range of peptide substrates that need to be handled by the intestinal peptide transporters. It is highly possible that other families of peptide transporters will be discovered in the near future.

CONCLUSIONS

Studies on the molecular basis of peptide transporters are helping to elucidate the number of peptide transporters, their mechanism of action as well as substrate specificity. Common features are being discovered among the various transporters involved in the peptide transport. Homologs of the human intestinal peptide transporter, hPepT1, have been found in other organs and other species. Knowledge gained from these transport systems will benefit oral drug delivery as well as other routes of administration. The broad substrate specificity of the intestinal peptide transport system may be utilized to improve the intestinal absorption of a wider array of administered drugs and apparently is not limited to peptidylmimetics. Further study of the systems for intestinal peptide transport is needed to fully understand and exploit this route of absorption for orally administered drugs.

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